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<b>(21) International Application Number:</b> PCT/US91/05323 <b>(22) International Filing Date:</b> 29 July 1991 (29.07.91)  <b>(30) Priority data:</b> 559,173                      27 July 1990 (27.07.90)                      US  <b>(71) Applicant:</b> THE CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 55 Shattuck Street, Boston, MA 02115 (US).  <b>(72) Inventor:</b> YANKNER, Bruce, A. ; 8 Whittier Place, Apt. 11B, Boston, MA 02114 (US).  <b>(74) Agent:</b> FREEMAN, John, W.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, PL, SE (European patent), SU.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> TACHYKININ AGONISTS FOR TREATMENT OF ALZHEIMER'S DISEASE  <b>(57) Abstract</b>  Method for treatment of a disease in a patient characterized by accumulation of $\beta$ -amyloid. The method includes identifying a patient potentially suffering from such a disease and contacting a neuron of the patient with a therapeutically effective amount of a tachykinin agonist. Methods for screening for compounds useful for treating such a disease are also disclosed.		

# + DESIGNATIONS OF "SU"

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## TACHYKININ AGONISTS FOR TREATMENT OF ALZHEIMER'S DISEASE

Background of the Invention

This invention was made with funding from the U.S. government; the U.S. government has rights in the invention.

This application is a continuation-in-part of my co-pending, commonly owned application, U.S.S.N. 07/559,173, filed July 27, 1990, which is hereby incorporated by reference.

This invention relates to treating diseases characterized by an undesirable buildup of  $\beta$ -amyloid protein (" $\beta$ -amyloid").

$\beta$  amyloid is a well characterized protein that is the primary constituent of senile plaques and cerebrovascular deposits in Alzheimer's disease and Down's syndrome. The form of  $\beta$  amyloid that is most prevalent in senile plaques contains 40-42 amino acids, but various other  $\beta$  amyloid forms are known, ranging from 28-43 amino acids in length.

$\beta$ -amyloid protein is encoded as part of a message that encodes a much larger precursor (the amyloid precursor protein, APP), carboxy terminal fragments of which are neurotoxic to hippocampal neurons in culture. Yankner et al., 245 Science 417, 1989 Whitson et al., 243 Science 1488, 1989, however, describe a peptide homologous to  $\beta$ -amyloid which increases the survival of young undifferentiated hippocampal neurons in cell culture.

Patients with Alzheimer's disease exhibit reduced levels of neurotransmitter peptides: Beal et al., Science (1985) 229:289-291 (receptors for somatostatin); Davis et al., Nature (1980) 288:279-280 and Rossor et al., Neurosci. Ltrs. (1980) 20:373-377 (somatostatin); Whitehouse, et al.

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Anal. Neurol. (1981) 10:122-126 (acetyl choline); Quigley et al., Neurosci (1986) 17:70a and Quigley et al. (1991) Neurosci 41:41-60 (Substance P); Adolfsson et al. (1978) In "Alzheimer's Disease, Senile Dementia and Related Disorders (Aging)" Ed. Katzman et al., Vol. 7, pp. 441-451 New York, Rauer (noradrenalin).

#### Summary of the Invention

Applicant has discovered that  $\beta$ -amyloid protein which accumulates abnormally in the brain is neurotoxic, and the deleterious effects of  $\beta$ -amyloid on neurons are selectively controlled or reversed by tachykinin agonists (e.g., those peptides having sequence similarity to specific tachykinins as discussed below). Accordingly, in a first aspect, the invention features a method for treatment or prophylaxis of neuronal accumulation of  $\beta$ -amyloid. The method includes identifying a patient potentially suffering from (or at risk for) such accumulation, and administering to the patient a therapeutically effective amount of a tachykinin agonist (defined below).

In preferred embodiments, the tachykinin agonist comprises a polypeptide region homologous to (e.g., a sequence at least three and preferably five or more amino acids in length from) substance P, physalaemin or neurokinin B. Specifically preferred agonists are Substance P, physalaemin and neurokinin B or derivatives (fragments or conservatively substituted sequences from) those three neurotransmitters. Useful tachykinin agonists and other therapeutics can also be identified by their ability to counteract  $\beta$ -amyloid accumulation on sensitive cells. Derivatives of Substance P (particularly N-terminal or C-terminal fragments or other derivatives) are particularly preferred, for example N-terminal Substance P fragments containing at least residues 1-4, or C-terminal fragments

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including amino acid residues 7-11. A particularly useful tachykinin agonist for use in the invention is the Substance P N-terminal fragment consisting of amino acid residues 1-7 ("Sub P [1-7]"). Other specific preferred fragments and derivatives are discussed below.

Specific Substance P derivatives or fragments, as well as other tachykinin agonists and other therapeutics for  $\beta$  amyloid associated diseases can be identified by their ability to reduce the neurotoxic effect of a neurotoxic  $\beta$ -amyloid related polypeptide on cultured neurons, neuronal cell lines or other  $\beta$ -amyloid sensitive cells. Accordingly, a second aspect of the invention features methods for identifying compounds (such as tachykinin agonists) useful for treatment of diseases (such as Alzheimer's disease or Down's syndrome) that are characterized by accumulation of  $\beta$ -amyloid.

One such method includes providing a potentially therapeutic compound, providing a neurotoxin comprising  $\beta$ -amyloid or a derivative polypeptide which is neurotoxic and determining whether the potentially therapeutic compound reduces the neurotoxic effect of the neurotoxin on a cell that is sensitive to the neurotoxin. Preferred cells for use in this method are, e.g., primary neurons or cultured neuronal cell lines including cells of tissues that developmentally derive from nervous tissue, such as brain tissue and adrenal medulla. Other cell lines that respond to the  $\beta$ -amyloid polypeptide can be used. A reduction of  $\beta$ -amyloid related neurotoxicity by the potentially therapeutic compound is indicative that the compound is useful for treatment of the disease.

A second such method comprises providing the potentially therapeutic compound in combination with a cell-surface accumulating  $\beta$ -amyloid peptide, and determining

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whether accumulation of the  $\beta$ -amyloid peptide on the cell is reduced by the compound. Reduction of the  $\beta$ -amyloid peptide accumulation is indicative that the compound is useful for treatment of the disease. Preferably the accumulation of  $\beta$ -amyloid peptide on the cell is determined by immunohistochemical staining, e.g., using a  $\beta$ -amyloid antibody or by staining with thioflavin S or congo red. Alternatively, accumulation can be measured by measuring uptake of a radiolabeled amyloid into cell-surface amyloid accumulations.

Other preferred embodiments of the second aspect of the invention include administering the  $\beta$ -amyloid-related polypeptide to a sensitive cell and measuring the neurotoxic effect of the polypeptide on the cell; administering the potential therapeutic (e.g. a tachykinin agonist as described above) in combination with the neurotoxin to the cell and measuring the neurotoxic effect of that combination; and comparing the neurotoxic effects of the neurotoxin alone with that of the combination.

While cultured cells can be used in the second aspect of the invention, it is also possible to conduct in vivo determinations by intracerebral injection into a mammal (e.g. rodents or primates) of the neurotoxin, in the presence or absence of the potential therapeutic. One particular embodiment of the second aspect of the invention features transgenic animals engineered to develop amyloid-related pathologies (e.g., by the introduction of DNA effecting overexpression of  $\beta$ -amyloid). The potential therapeutic is administered (e.g. by intracerebral injection, or by system administration--oral or i.v.) to determine whether neuronal loss is reduced.

Also we have recognized that the entire  $\beta$ -amyloid protein itself, e.g.,  $\beta$  amyloid residues 1-43 without the

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flanking APP sequences, is neurotoxic, and, indeed, polypeptides comprising residues 29-35 alone of the  $\beta$ -amyloid protein are neurotoxic. This recognition is beneficial in the second aspect of the invention in which

5 antagonists of such toxicity (tachykinins or other compounds) are identified as useful for treating disease characterized by neuronal accumulation of  $\beta$ -amyloid. Specifically, a neurotoxic polypeptide comprising at least residues 29-35 (preferably 25-35) of the  $\beta$ -amyloid protein

10 (without APP sequences flanking the full 43-residue  $\beta$ -amyloid protein) is administered to a sensitive cell in conjunction with the compound being tested as a toxicity antagonist to determine whether the compound reduces the effect of the neurotoxicity (e.g. reduces death) or reduces

15 accumulation on the sensitive cell. This method has the advantage that the neurotoxin can be chemically synthesized rather than being produced by expression in an engineered cell, which, as a practical matter, is the method for producing larger derivatives of APP. See generally, PCT

20 WO89/05138, hereby incorporated by reference. This aspect of the invention generally serves as a screening procedure for therapeutics related to diseases characterized by excessive neuronal deposits of  $\beta$ -amyloid.

It should be noted that we recognize that neurotoxic

25  $\beta$ -amyloid related polypeptides can be neurotrophic at earlier stages of neuronal differentiation. The phenomenon of interest in the invention, however, is the effect of the neurotoxicity of such polypeptides to mature neurons.

Other features and advantages of the invention will

30 be apparent from the following description of the preferred embodiments, and from the claims.

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### Description of the Preferred Embodiment

The drawings will first briefly be described.

#### Drawings

Figs. 1A and 1B are graphical representations of the neurotrophic and neurotoxic effects of  $\beta$ -amyloid on hippocampal neurons;

Fig. 2 is a graphical representation of the  $\beta$ -amyloid concentration dependence of the neurotrophic and neurotoxic responses;

Fig. 3 is a representation of the amino acid sequence of  $\beta$ -amyloid and various tachykinin neuropeptides; and

Fig. 4 is a graphical representation of the effect of tachykinins on the trophic and toxic responses to  $\beta$ -amyloid.

#### Tachykinin Agonists

Tachykinin agonists useful in this invention can be identified by any of a number of techniques, specific examples of which are provided below. In general, tachykinin agonists are those polypeptides which are able to overcome or reduce the neurotoxic effect of  $\beta$ -amyloid related polypeptides. An example of such a neurotoxic effect is also provided below. The tachykinin agonists are able to reduce, or even prevent or reverse, the neurotoxic effects of neurotoxic  $\beta$ -amyloid related polypeptide. In particular, they are able to reduce the neurotoxic and effects of the intact  $\beta$ 1-40 peptide or of a peptide that includes the 11 amino acid portion of the  $\beta$ -amyloid protein shown in Fig. 3, between amino acids 25 and 35 inclusive: GSNKGAIIGLM. (As mentioned above, even the region 29-35 is adequate to generate neurotoxicity that can form the basis of an assay according to the invention.)



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Potentially useful tachykinin agonists are those which have significant sequence similarity to either substance P, physalaemin, or neurokinin B. That is, they have either conservative amino acid substitutions at one or more positions. Preferably there are only one or two non-conservative substitutions in the proposed therapeutics, but the invention at its broadest level covers polypeptides with substantially more (e.g. 50%) substitutions to a proven tachykin (e.g. substance P, physalaemin or neurokinin B) only one or two of the amino acids of these compounds. As discussed above, useful agonists can be readily identified from such homologous compounds by standard techniques, examples of which are provided below.

Tachykinin agonists of this invention inhibit the toxic effects of  $\beta$ -amyloid-related proteins in the assay given below. These agonists are thought to interact at the receptor for substance P or the receptor for  $\beta$  amyloid within the brain or with  $\beta$  amyloid itself; thus, tachykinin agonists which interact strongly at any one of these sites are particularly useful in this invention. Such agonists can again be identified by standard procedure by simply measuring their binding capacity for substance P receptors, for example, in a radioreceptor assay using substance P as a competitive binding agent for a substance P receptor.

There follows an example of a method by which the capacity of a potential tachykin agonist to inhibit the deleterious effects of  $\beta$  amyloid can be measured. This example is provided to illustrate, not to limit the invention; those of ordinary skill in the art can readily determine other equivalent methods by which to measure the neurotoxic and neurotropic effect of  $\beta$ -amyloid-related proteins, the reversal of such effect, or the inhibition of such effect by useful tachykinin agonists of this invention.

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In this example, the polypeptide corresponding to the first 40 amino acids of  $\beta$ -amyloid ( $\beta$ 1-40) was synthesized, purified, and the primary sequence confirmed (see Fig. 3). Referring to Fig. 1, the effects of the  $\beta$ 1-40 polypeptide on hippocampal neurons was measured. As mentioned above, both neurotrophic and neurotoxic effects were observed, depending on the stage of neuronal development. In Fig. 1A, 20 $\mu$ M  $\beta$ 1-40 was added to hippocampal neurons at plating to result in an early increase (0-2 days, neurotrophic) followed by a decrease (3-5 days, neurotoxic) in neuronal cell number. Thus, addition of  $\beta$ 1-40 to primary rat E18 hippocampal cultures at the time of subplating results in a significant increase in the number of pyramidal neurons during the first 2 days in culture. After 3 days in culture, however, there is a marked decline in neuronal cell number in cultures treated with  $\beta$ 1-40, and by 4-5 days the number of pyramidal neurons in  $\beta$ 1-40 treated cultures was significantly less than in control cultures.

Referring to Fig. 1B, 20 $\mu$ M  $\beta$ 1-40 was added to hippocampal neurons of different ages in culture, and the number of neurons determined 24 hours later.  $\beta$ 1-40 had a trophic effect on young neurons (values greater than 100% on days 0-2) and a toxic effect on older neurons (values less than 100% on days 3-5). The dashed line in the drawing indicates the transition from the trophic to toxic response. Thus, when  $\beta$ 1-40 is added at the time of plating (day 0) there is a significant increase in 24 hour neuronal survival relative to control values. This trophic effect progressively declines when  $\beta$ 1-40 is added during the next two days in culture. If  $\beta$ 1-40 is added to older cultures (3 days or later) there is an opposite effect, with a decline in 24 hour neuronal survival relative to controls. Control

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cultures remain viable and showed only a small change in neuronal survival using neurons up to 5 days old. These data show that  $\beta$ 1-40 is neurotrophic when added during the early period of neuronal differentiation (days 0-2) and neurotoxic to older more differentiated neurons, more similar to an adult state.

Referring to Fig. 2, the neurotrophic and neurotoxic effects of  $\beta$ 1-40 was separately assayed by adding  $\beta$ 1-40 at day 0 and day 4, respectively, and determining 24 hour neuronal survival. The  $\beta$ 1-40 concentration dependency of the neurotrophic and neurotoxic effects is shown in Fig. 2. The neurotoxic response requires about 1000-fold higher concentration of  $\beta$ 1-40 than the neurotrophic response. The trophic response was determined by adding  $\beta$ 1-40 at the indicated concentrations to neurons at the time of plating (day 0); the toxic response was determined by adding  $\beta$ 1-40 to neurons at day 4 in culture. Values were normalized to the maximum  $\beta$ 1-40-induced increase in neuronal cell number at day 0 (100% trophic response) and the maximum  $\beta$ 1-40 induced decrease in neuronal cell number at day 4 (100% toxic response). The trophic response was detected at very low levels of  $\beta$ 1-40 with an  $EC_{50}$  of 0.06nM. The toxic response required about 1000-fold higher concentrations of  $\beta$ 1-40; it was first detected at 40nM, with an  $EC_{50}$  of about 100nM.

The APP domain responsible for neurotrophic and neurotoxic effects was determined by assaying overlapping peptides spanning the entire  $\beta$ -amyloid precursor sequence (see Fig. 3 and table 1). The figures in the table were determined by treating hippocampal neurons at the time of cell plating, or at 4 days in culture, with the indicated peptides to measure the early trophic or late toxic

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responses, respectively, one day later. Values were normalized to the mean day 1 response (trophic response) and day 5 decrease (toxic response) in neuronal cell number observed for  $\beta$ 1-40 (100% response). Peptide concentrations were at 20  $\mu$ M except where indicated otherwise, and added directly to cell cultures. The values shown in the table are the mean  $\pm$  the standard error of the mean using between 10 and 20 measurements for each peptide. The primary sequences of the designated  $\beta$ -amyloid sequences are shown in Fig. 3.

TABLE 1

	<u>Peptide</u>	<u>% Trophic Response</u>	<u>% Toxic Response</u>
	$\beta$ 1-40	100 $\pm$ 6	100 $\pm$ 7
	$\beta$ 1-38	109 $\pm$ 10	97 $\pm$ 9
15	$\beta$ 1-28 20 $\mu$ M	0 $\pm$ 5	0 $\pm$ 6
	$\beta$ 28 100 $\mu$ M	29 $\pm$ 10	55 $\pm$ 11
	$\beta$ 1-16	0 $\pm$ 8	0 $\pm$ 10
	$\beta$ 17-28	0 $\pm$ 4	0 $\pm$ 8
	$\beta$ 25-35	100 $\pm$ 6	117 $\pm$ 12
20	$\beta$ 32-42	0 $\pm$ 10	0 $\pm$ 7
	APP576-695	0 $\pm$ 4	0 $\pm$ 7
	Glucagon	0 $\pm$ 3	0 $\pm$ 11
	Substance P	16 $\pm$ 8	0 $\pm$ 7
	Physalaemin	0 $\pm$ 4	0 $\pm$ 7
25	Eledoisin	0 $\pm$ 11	0 $\pm$ 6
	[D-Pro <sup>2</sup> , D-Trp <sup>7,9</sup> ]- substance P	125 $\pm$ 11	117 $\pm$ 13
	[D-Arg <sup>1</sup> , D-Trp <sup>7,9</sup> , Leu <sup>1</sup> ]-substance P		
30	(spantide)	120 $\pm$ 8	118 $\pm$ 10
	Spantide + Substance P	0 $\pm$ 8	26 $\pm$ 7

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81-38 elicited the same activity as 81-40. 81-28 showed some early neurotrophic and late neurotoxic activity, but was much less potent than 81-40 (Table 1). 81-16 and 817-28 showed no trophic or toxic activity at equimolar concentrations to 81-40. 817-28 showed similar activity to 81-28 at higher concentrations. The 825-35 peptide showed the same neurotrophic and neurotoxic activity as 81-40. 834-42 as inactive. A peptide corresponding to the carboxyterminal 20 amino acids of the amyloid precursor protein (APP676-695) and glucagon, a 28 amino acid peptide possessing  $\beta$ -pleated sheet structure similar to that of  $\beta$ -amyloid, were both inactive. Thus, a sufficient portion of the functional domain of  $\beta$ -amyloid required for the toxic effects is contained in the 825-35 sequence and even in the  $\beta$  29-35 sequence. The dose response relationship shown in Fig. 2 for 81-40 was also observed for 825-35.

825-35 has 73% homology to eledoisin, including conservative changes, and 56% homology to the other tachykinins (Fig. 3). The region of greatest homology is in the carboxyterminal amino acids of the tachykinin sequence which is known to be required for high affinity tachykinin receptor binding and biological activity. Payan 40 Ann Rev. Med. 341, 1989.

Various tachykinins were tested for their effects on hippocampal neuronal survival. Exogenous substance P, eledoisin and physalaemin had no effect on early or late neuronal survival (Table 1). Tachykinin antagonists were also tested. The potent tachykinin antagonists [D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>]-substance P and [D-Arg<sup>1</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]-substance P (spantide) showed significant early neurotrophic and late neurotoxic effects which could be reversed by the addition of substance P (Table 1). The effects of tachykinin

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antagonists closely mimicked those of  $\beta$ 1-40 with respect to the time course and magnitude of changes in neuronal survival (Table 1).

5 Since  $\beta$ -amyloid appears to have the same effects as tachykinin antagonists, the following experiment was performed to determine whether its activity could be reversed by tachykinin agonists. Tachykinin peptides were added along with  $\beta$ 1-40 to hippocampal neurons at the time of plating to assay the trophic effect. Similarly, the toxic  
10 effect was assayed by adding these compounds after 4 days to older neurons in culture.  $\beta$ 1-40 was maintained at  $20\mu\text{M}$  at the time of cell plating and the trophic response determined 1 day later. The results of such experiments are shown in Figs. 4A and 4B.

15 Substance P and physalaemin almost completely reversed the early trophic and late toxic responses to  $\beta$ 1-40. Values were normalized to the mean day 0 trophic and day 4 toxic responses to  $\beta$ 1-40 alone (100%); substance P and physalaemin acted in a dose dependent manner. Neurokinin B  
20 partially reversed the activity of  $\beta$ 1-40, but was less potent than substance P and physalaemin. Neurokinin A, eledoisin and kassinin did not show significant effects in the concentration ranges tested. Thus, the effects of  $\beta$ -amyloid are selectively reversed by specific tachykinin  
25 neuropeptides.

An alternative method for identifying useful therapeutic that reverse the neurotoxicity of  $\beta$  amyloid involves intracerebral injection of  $\beta$  amyloid. Coadministration of the potential therapeutic compound is  
30 tested for its ability to prevent an intracerebral neurotoxic response to  $\beta$  amyloid alone or in combination with neurotrophic factors. For example, test animals such as rats or monkeys can be injected and, after treatment, can

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be autopsied using antibodies specific for Alzheimer's Disease, e.g. antibodies to Tau protein, A68 proteins, or ubiquitin.

Still another method for identifying useful  
5 therapeutic involves measuring the accumulation of a  $\beta$  amyloid peptide (as described above) on the surface of sensitive cell. Generally, the cells that are sensitive to  $\beta$ -amyloid toxicity, as mentioned above, can be used in the  $\beta$  amyloid "accumulation" assay. The measurement can be made  
10 by using antibodies to  $\beta$  amyloid or by staining with thioflavin S or congo red and measuring fluorescence, or green birefringence, respectively.

One cell line in particular that can be used in this aspect of the invention is PC-12, a cell line derived from a  
15 tumor (pheochromocytoma) of the achenal medulla.

Still another method of identifying useful therapeutics involves the use of transgenic mammals that have been genetically engineered to overproduce  $\beta$  amyloid. See Quon et al. Nature (1991) 352:239-241 regarding such a  
20 transgenic mouse. See also Wirak et al. Science (1991) 253:323-325; and Marx, Science (1991) 253:266-267. Neuronal death is measured as a control, and the potential therapeutic is administered. A determination (e.g. autopsy and staining) is then made on the mouse's neurons to  
25 ascertain amelioration of the neuronal death.

Other tachykinin agonists suitable for use in the invention include fragments of Substance P which counteract the neurotoxicity of  $\beta$  amyloid peptides in the above-described assays.

30 As one specific example, the Substance P fragment comprising amino acid residues 1-7 ("Substance P (1-7)") is suitable for use in the invention. The sequence of Substance P (1-7) is RPKPQQF. Advantageously, Substance P

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(1-7) has substantial therapeutic efficacy, but it has less biological activity than Substance P on peripheral organs (i.e., organs outside the central nervous system), thereby reducing potential side effects to patients.

5       Without limiting the scope of the invention I illustrate it with the following additional specific fragments of Substance P: Sub P (6-11)--QFFGLM; and Sub P (7-11)--FFGLM; Sub P (1-4)--RPKP, Sub P (5-11)--QFFGLM. Substance P (2-11)--PKPQQFFGLM; Substance P (4-11)--  
10   PQQFFGLM; and Substance P (1-9)--RPKPQQFFG.

      Still other tachykinin antagonists useful in the invention are tachykinins in which amino acid substitutions have been made. For example Substance P may be modified by substituting Tyr for Phe in position 7, or ethionine for Gly  
15   in position 11 or nor-leucine for Gly in position 11, or Ile for Phe at position 8. Other analogues with relevant activity are Arg<sup>1</sup>→pGlu; Phe<sup>7</sup>→Met.

      Substance P is somewhat stable on passage through the circulatory system. Certain active analogs, e.g., Pro  
20   in place of Arg (1), or Lys (3) reduce enzymatic degradation of Substance P and retain activity. Similarly, Substance P analogs with one or more of the following substitutions (Gln<sup>5</sup>→Glu, Phe<sup>8</sup>→MePhe, and Gly<sup>9</sup>→MeGly) are more resistant to degradation.

25       Other analogues include alkyl (e.g., methyl, ethyl, or propyl esters of Substance P (i.e., the terminal Met-NH<sub>2</sub> is Met-OCH<sub>3</sub> or Met-OCH<sub>2</sub>CH<sub>3</sub> or Met-O-CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>) or the free acid of substance P in which the terminal Met-NH<sub>2</sub> is (Met-OOH). Still other analogs of Substance P are those with the  
30   following substitutions: Ala<sup>9</sup> D-Ala<sup>9</sup> Sar<sup>9</sup> Pro<sup>9</sup> or D-Pro<sup>9</sup>; MeLeu<sup>10</sup> or Pro<sup>10</sup>; MeMet<sup>11</sup> or Pro<sup>11</sup>.



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Still other useful analogs are cyclic peptides in which cysteine or homocysteine is introduced particularly at 5, 9 and 5, 10 and 5, 11 positions in two locations to permit disulfide bridge cyclization. Useful compounds

5 include [Cys<sup>5,9</sup>]SP; [Hcy<sup>5,9</sup>]SP; [D-Cys<sup>5</sup>, Hcy<sup>10</sup>]SP; [Cys<sup>5,11</sup>]SP; [Hcy<sup>5,11</sup>]SP.

Other useful cyclic analogs of Substance P are given below:

10 [D-Cys<sup>5</sup>, Cys<sup>8</sup>]SP; [D-Cys<sup>5</sup>, Cys<sup>7</sup>]SP; [D-Cys<sup>3</sup>, Cys<sup>6</sup>]SP; [Cys<sup>3,6</sup>]SP; [Cys<sup>3,6</sup>, Tyr<sup>8</sup>]SP; [Cys<sup>3,6</sup>, Val<sup>8</sup>]SP; [Cys<sup>3,6</sup>, Tyr<sup>8</sup>, Ala<sup>9</sup>]SP; [Cys<sup>3,6</sup>, Tyr<sup>8</sup>, Pro<sup>9</sup>]SP; [Cys<sup>3,6</sup>, Tyr<sup>8</sup>, Pro<sup>10</sup>]SP.

Other useful analogs of Neurokinin B (NKB) or Substance P are:

15 [Cys<sup>7,5</sup>]NKB; [Me-Val<sup>7</sup>]NKB; [Pro<sup>7</sup>]NKB; [pGlu<sup>6</sup>, Pro<sup>9</sup>]SP(ε-11)<sup>+</sup>; [pGlu<sup>5</sup>, MePhe<sup>8</sup>]-Sub P (5-11)] = GFFMePheLM  
[Nle<sup>11</sup>]-SP (7-11) -- FFGLNle  
[Tyr<sup>7</sup>]-SP (7-11) -- YFGLM  
[Eth<sup>11</sup>]-SP (7-11) -- FFGLEth.

20 Finally, the following Substance P analogs are suitable for use in the invention:

[pGlu<sup>5</sup>, MePhe<sup>8</sup>, Sar<sup>9</sup>]-Sub P (5-11)  
[Asp<sup>5,6</sup>, MePhe<sup>8</sup>] Sub P (5-11) (Senktide Analog)  
[N-Acetyl-Arg<sup>6</sup>, MePhe<sup>8</sup>]-Sub P (6-11) (Senktide)  
[pGlu<sup>6</sup>, Pro<sup>9</sup>]-Sub P (6-11) (Septide)  
25 [p-Chloro-Phe<sup>7,8</sup>]-Sub P  
[Sar<sup>9</sup>, Met(O<sub>2</sub>)<sup>11</sup>]-Sub P  
[D-Ala<sup>4</sup>]-Sub P (4-11)].

Still further analogs are those described in Scandrett, U.S. Patent 3,862,114, which is hereby  
30 incorporated by reference.

#### Examples

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Substance P and various analogs were tested for therapeutic potency. Specifically, as a control, primary hippocampal neurons were incubated with the  $\beta$ -amyloid peptide ( $\beta$ 1-40) as generally described above. The control showed 53% neuronal loss after 24 hours of incubation. In the presence of 10 $\mu$ g/ml of Substance P, the toxicity of ( $\beta$ 1-40) was completely blocked. The substance P analogs were also added to separate cultures at a concentration of 10 $\mu$ g/ml. The ability of the analogs to block neuronal toxicity is expressed below:

	<u>Peptide</u>	<u>Therapeutic Efficacy (%)</u>
	Sub P	100
	Sub P (1-7)	82
	Sub P (6-11)	65
15	Sub P (7-11)	41
	[Nle <sup>11</sup> ]-Sub P	77
	Sub P, Methyl Ester	71
	Sub P, Free Acid	53
	[Cys <sup>3,6</sup> , Tyr <sup>8</sup> , Pro <sup>9</sup> ]-Sub P	53
20	[pGlu <sup>5</sup> , Me-Phe <sup>8</sup> , Sar <sup>9</sup> ]-Sub P (5-11)	41
	[Succinyl-Asp <sup>6</sup> , Me-Phe <sup>8</sup> ]-Sub P (6-11) (Senktide)	47

The above described analogs generally can be purchased (e.g., from Peninsula Lab, Inc., San Carlos, CA and Bachem Fine Chemicals, Torrence CA) and therapeutic compositions can be prepared by standard techniques for purification and formulation into a pharmaceutically acceptable vehicle. Those compounds which are not readily available commercially can be prepared by solid phase

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synthesis: Merrifield, J. Am. Chem. Soc. 85:2149 (1963). Other synthetic techniques are disclosed in Chipkin et al., Arch. Int. Pharmacodyn. "SP and Analogs" 240:193-202 (1979); Eison et al., Science (1982) 188-190 "Substance P Analog, DiMe-C7: Evidence For Stability in Rat Brain and Prolonged Central Action"; Bar-Shavitt et al., Biochem. Biophys. Res. Com. 94:1445-1451 (1980); Lavielle et al., Biochem. Pharm. 37:41-49 (1988).

In as much as it is clearly possible to inhibit binding between Substance P and its receptor (the NK1 receptor) using nonpeptides [see Snider et al. Science 251: 435-437 (1991)], nonpeptide agonists of Substance P are also within the scope of this invention.

#### Use

Tachykinin agonists of this invention are useful for treatment of diseases characterized by accumulations of  $\beta$ -amyloid within a central nervous system. Such diseases include Alzheimer's disease, Down's syndrome, and the syndromes of hereditary cerebral hemorrhage with amyloidosis and non-inherited congophilic angiopathy with cerebral hemorrhage. Patients who are at risk or who may be affected by such diseases can be generally identified by procedures well known to those of ordinary skill in the art, including external manifestations of such diseases, such as declined mental efficiency and focal neurological deficits. They may also be characterized by detection of  $\beta$ -amyloid accumulation as described by Joachium et al. 341 Nature 226, 1989. Once characterized, these patients can be treated by administering a tachykinin antagonist of this invention in an amount sufficient to reduce symptoms of the disease, or to inhibit progress of the disease. The amount of agonist to be administered will vary dependent upon the agonist, and can be determined by standard procedures. For any

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particular agonists, it is expected that a useful dose will be in the range of one nanomolar to one micromolar agonist, administered with a physiologically acceptable carrier either systemically or directly to the central nervous system. The agonist may be administered orally or by intravenous, subcutaneous or intramuscular injection directly into the patients' tissues. The peptides may also be modified to enhance their absorption directly into the body, and thus may be administered topically. The agonist may be administered directly into the brain by an indwelling catheter or pump device which delivers the agonist into the cerebral ventricles or intrathecal spaces.

One specific drug delivery system relies on redox interconversion of dihydropyridine and a pyridinium salt carrier. See Sodor et al. Science, 214:1370 et seq. (1981). That system describes attaching the pyridinium salt carrier by a dehydration reaction to the amino terminus of the peptide to be delivered. The carboxyl terminus is protected by methanol condensation with the free acid to produce the methyl ester.

Following intravenous injection, the compound will cross the blood-brain barrier and will be distributed throughout the body, including the brain. Drug removal occurs as the dihydropyridine carrier is oxidized to the original charged pyridinium quaternary salt, which is eliminated. Enzymatic cleavage of the carrier drug combination traps the drug in the central nervous system, resulting in sustained delivery of substance P to neurons.

Substance P itself is known to cross the blood-brain barrier, (See, e.g., Banks et al. Res. Bul., 15:287-292 (1985)) and therefore may be administered systemically. It is also known to be somewhat stable to protease degradations. In general, less charged substance P analogs

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(such as [Nle<sup>2</sup>]-Sub P and Sub P-methyl ester therefore, also cross the blood-brain barrier. Such compounds can also be administered systemically, e.g., orally, intravenously, or intramuscularly. In general, the ability of therapeutic

- 5 Substance P fragments and derivatives to cross the blood-brain barrier can be measured by the method described by Banks et al. Those with suitable ability to cross can be administered systemically, preferably orally.

Other embodiments are within the following claims.

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Claims

- 1           1. A method for treatment or prophylaxis of  
2 neuronal accumulation of  $\beta$ -amyloid in a patient, comprising  
3 the steps of:  
4           identifying a patient at risk for said accumulation;  
5 and  
6           administering to neurons of said patient a  
7 therapeutically effective amount of a tachykinin agonist.
- 1           2. The method of claim 1 wherein said tachykinin  
2 agonist comprises a polypeptide region homologous to  
3 Substance P, physalaemin or neurokinin B.
- 1           3. The method of claim 1 wherein said tachykinin  
2 agonist is substance P, physalaemin, neurokinin B, or a  
3 derivative thereof.
- 1           4. The method of claim 1 wherein said tachykinin  
2 agonist is capable of reducing the neurotoxic effect of a  $\beta$ -  
3 amyloid-related polypeptide on cultured neurons or neuronal  
4 cell lines.
- 1           5. The method of claim 1 wherein said tachykinin  
2 agonist is Substance P or a fragment or derivative thereof.
- 1           6. The method of claim 5 wherein said tachykinin  
2 agonist is an N-terminal or a C-terminal fragment of  
3 Substance P.
- 1           7. The method of claim 5 wherein said tachykinin  
2 agonist is an N-terminal fragment of Substance P comprising  
3 amino acid residues 1-4.

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1           8. The method of claim 7 wherein said tachykinin  
2 agonist is Substance P (1-7).

1           9. The method of claim 5 wherein the tachykinin  
2 agonist is a Substance P fragment selected from the group  
3 consisting of:

- |                                  |                       |
|----------------------------------|-----------------------|
| 4           a) Substance P (1-9) | f) Substance P (2-11) |
| 5           b) Substance P (1-8) | g) Substance P (3-11) |
| 6           c) Substance P (1-7) | h) Substance P (4-11) |
| 7           d) Substance P (1-6) | i) Substance P (5-11) |
| 8           e) Substance P (1-5) | j) Substance P (6-11) |
| 9           f) Substance P (1-4) | l) Substance P (7-11) |

1           10. The method of claim 1 wherein the tachykinin  
2 agonist is a Substance P derivative selected from the group  
3 consisting of:

- |   |  |
|---|--|
| 4           a) Substance P [Tyr <sup>7</sup> ]  | b) Substance P [Pro <sup>9</sup> ]                                 |
| 5           c) Substance P [Ethionine <sup>11</sup> ]                                     | d) Substance P [D-Pro <sup>9</sup> ]                               |
| 6           e) Substance P [Nle <sup>11</sup> ]   | f) Substance P [Me-Leu <sup>10</sup> ]                             |
| 7           g) Substance P [Ile <sup>8</sup> ]  | h) Substance P [Pro <sup>10</sup> ]                                |
| 8           i) Substance P [pGlu <sup>1</sup> ]   | j) Substance P [MeMet <sup>11</sup> ]                              |
| 9           k) Substance P [Met <sup>7</sup> ]  | l) Substance P [Pro <sup>11</sup> ]                                |
| 10          m) Substance P [Pro <sup>1</sup> ]  | n) Substance P [Me-Phe <sup>8</sup> ]                              |
| 11          o) Substance P (6-11)<br>12           [pGlu <sup>6</sup> , Pro <sup>9</sup> ] | p) Substance P (5-11)<br>[pGlu <sup>5</sup> , MePhe <sup>8</sup> ] |
| 13          q) Substance P [Pro <sup>3</sup> ]  | r) Substance P [Tyr <sup>7</sup> ]                                 |
| 14          s) Substance P [Glu <sup>5</sup> ]  | t) Substance P (7-11)[Tyr <sup>7</sup> ]                           |
| 15          u) Substance P [MePhe <sup>8</sup> ]  | v) Substance P (7-11)<br>16           [Eth <sup>11</sup> ]         |

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- |    |  |  |
|----|--|--|
| 17 | w) Substance P [MeGly <sup>9</sup> ]     | x) Substance P (7-11)  |
| 18 |  | [Nle <sup>11</sup> ]   |
| 19 | y) Substance P [alkyl ester]             | z) [pGlu <sup>5</sup> , MePhe <sup>8</sup> ,                       |
| 20 |  | Sar <sup>9</sup> ]-Sub P (5-11)                                    |
| 21 | aa) Substance P [free acid]              | bb) [Asp <sup>5,6</sup> , MePhe <sup>8</sup> ]                     |
| 22 |  | Sub P (5-11) (Senktide   |
| 23 |  | Analog)  |
| 24 | cc) Substance P [Ala <sup>9</sup> ]      | dd) [N-Acetyl-Arg <sup>6</sup> ,                                   |
| 25 |  | MePhe <sup>8</sup> ] Sub P (6-11)                                  |
| 26 |  | (Senktide)   |
| 27 | ee) Substance P [D-Ala <sup>9</sup> ]    | ff) [pGlu <sup>6</sup> , Pro <sup>9</sup> ] Sub P                  |
| 28 |  | (6-11) (Septide)   |
| 29 | gg) Substance P [Sar <sup>9</sup> ]      | hh) [Sar <sup>9</sup> , Met(O <sub>2</sub> ) <sup>11</sup> ] Sub P |
| 30 | ii) [p-Chloro-Phe <sup>7,8</sup> ] Sub P | jj) [D-Ala <sup>4</sup> ] Sub P (4-11)                             |

1           11. The method of claim 1 wherein the tachykinin  
 2 agonist is a cyclic Substance P derivative selected from the  
 3 group consisting of: [Cys<sup>5,9</sup>]SP; [Hcy<sup>5,9</sup>]SP; [D-Cys<sup>5</sup>,  
 4 Hcy<sup>10</sup>]SP; [Cys<sup>5,11</sup>]SP; [Hcy<sup>5,11</sup>]SP; [D-Cys<sup>5</sup>, Cys<sup>8</sup>]SP; [D-  
 5 Cys<sup>5</sup>, Cys<sup>7</sup>]SP; [D-Cys<sup>3</sup>, Cys<sup>6</sup>]SP; [Cys<sup>3,6</sup>]SP; [Cys<sup>3,6</sup>,  
 6 Tyr<sup>8</sup>]SP; [Cys<sup>3,6</sup>, Val<sup>8</sup>]SP; [Cys<sup>3,6</sup>, Tyr<sup>8</sup>, Ala<sup>9</sup>]SP; [Cys<sup>3,6</sup>,  
 7 Tyr<sup>8</sup>, Pro<sup>9</sup>]SP; [Cys<sup>3,6</sup>, Tyr<sup>8</sup>, Pro<sup>10</sup>]SP

1           12. The method of claim 1 wherein the tachykinin  
 2 agonist is a derivative of neurokinin B.

1           13. A method for identifying a potentially  
 2 therapeutic compound useful for treatment of disease  
 3 characterized by neuronal accumulation of  $\beta$ -amyloid  
 4 comprising the steps of:  
 5           providing a potentially therapeutic compound;  
 6           providing a neurotoxin comprising  $\beta$ -amyloid or a  
 7 derivative polypeptide which is neurotoxic; and



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8 determining whether said potentially therapeutic  
9 compound reduces the neurotoxic effect of said neurotoxin on  
10 a cell that is sensitive to the neurotoxin; wherein a  
11 reduction of said effect by said potential therapeutic is  
12 indicative that said potential therapeutic is useful for  
13 treatment of the disease.

1 14. The method of claim 13 wherein said cell is a  
2 primary neuron or a neuronal cell line, and said determining  
3 step comprises:

4 administering said neurotoxin to said cell and  
5 measuring the neurotoxic effect of said neurotoxin on said  
6 cell; and

7 administering said potential potential therapeutic  
8 and said polypeptide to said cell.

1 15. The method of claim 13 wherein said determining  
2 step comprises:

3 intracerebral injection of said neurotoxin into a  
4 mammal.

1 16. The method of claim 13 wherein said determining  
2 step comprises administering said neurotoxin to said  
3 sensitive cell and measuring the toxic effect of said  
4 neurotoxin on said cell; and administering said potential  
5 therapeutic compound and said neurotoxin to said cell.

1 17. The method of claim 13 wherein said determining  
2 step comprises:

3 a) administering said potentially therapeutic  
4 compound to a transgenic mammal, said mammal comprising  
5 engineered DNA effecting overexpression of  $\beta$ -amyloid protein  
6 so as to cause neuronal loss; and

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7           b) determining whether said neuronal loss is  
8 ameliorated by said potentially therapeutic compound.

1           18. A method for identifying an antagonist of  
2 neurotoxicity useful for treatment of disease characterized  
3 by neuronal accumulation of  $\beta$ -amyloid, said method  
4 comprising  
5           providing a potential neurotoxicity antagonist;  
6           providing a  $\beta$ -amyloid-related polypeptide comprising  
7 at least residues 29-35 of  $\beta$ -amyloid and lacking the APP  
8 sequences flanking the maximal 1-43  $\beta$ -amyloid sequence;  
9           administering said  $\beta$ -amyloid-related polypeptide to  
10 a neuron or neuronal cell line in conjunction with a  
11 potential neurotoxicity antagonist; and  
12           determining whether said potential neurotoxicity  
13 antagonist reduces the neurotoxic effect of said  $\beta$ -  
14 amyloid-related polypeptide on said neuron or neuronal cell  
15 line.

1           19. The method of claim 18 wherein said  $\beta$ -amyloid  
2 peptide comprises at least residues 25-35 of  $\beta$ -amyloid.

1           20. A method for identifying a compound useful for  
2 treatment of a disease characterized by neuronal  
3 accumulation of  $\beta$ -amyloid comprising the steps of:  
4           a) providing a potentially therapeutic compound;  
5           b) providing  $\beta$ -amyloid or a  $\beta$ -amyloid related  
6 peptide which accumulates on the surface of cells, and  
7           c) determining whether said potentially therapeutic  
8 compound reduces the accumulation of said  $\beta$ -amyloid or  $\beta$ -  
9 amyloid related peptide on said cells; wherein a reduction  
10 of accumulation in the presence of said potentially

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11 therapeutic compound is indicative that said compound is  
12 useful for the treatment of said diseases.

1           21. The method of claim 20 wherein  $\beta$ -amyloid  
2 accumulation on neuronal cells is determined by  
3 immunohistochemical staining.

1           22. The method of claim 20 wherein said  $\beta$ -amyloid  
2 accumulation is determining by binding of a radiolabeled  
3  $\beta$ -amyloid polypeptide.

1           23. The method of claim 20 wherein said  $\beta$ -amyloid  
2 accumulation is measured by indirect staining.

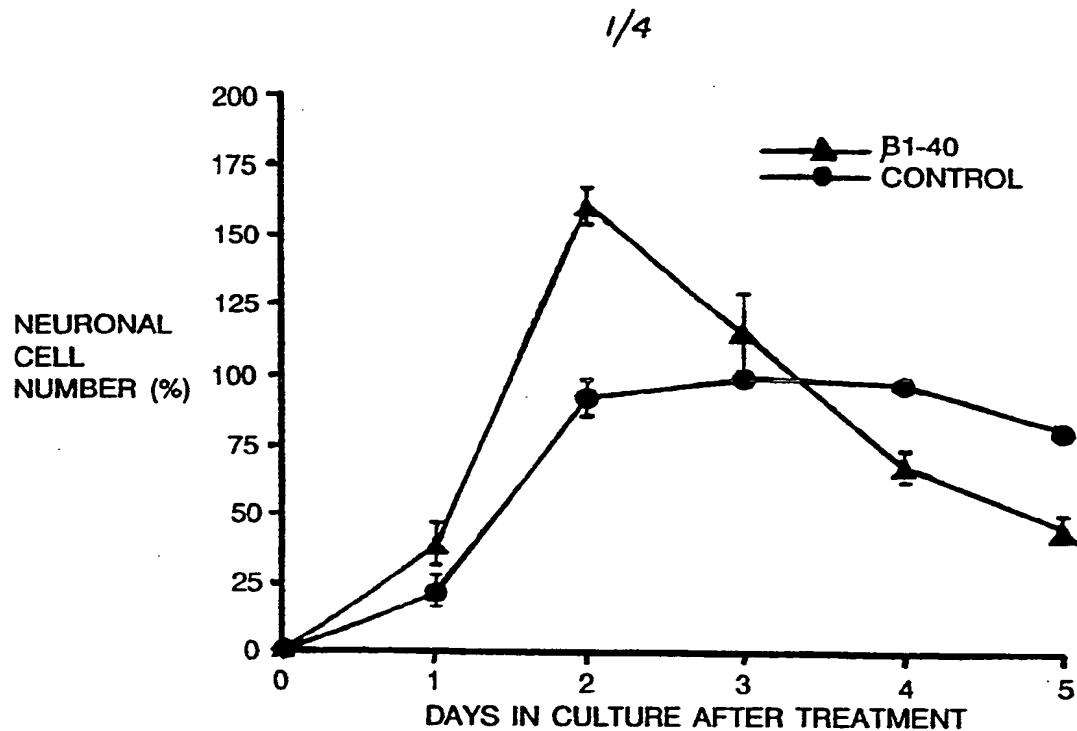


FIG. 1a

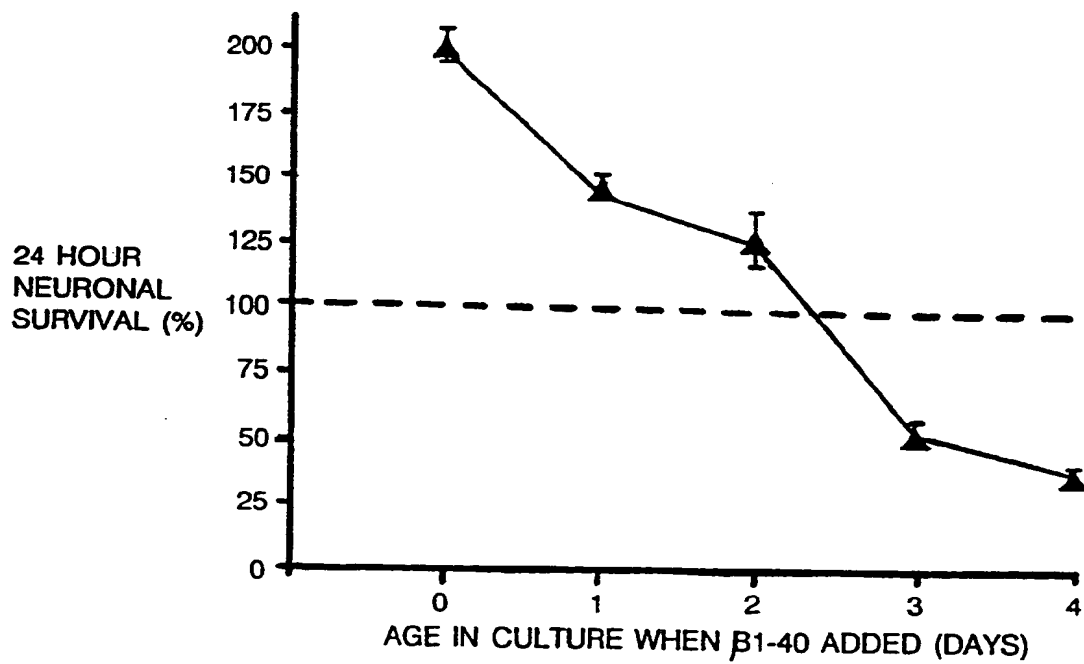


FIG. 1b

SUBSTITUTE SHEET

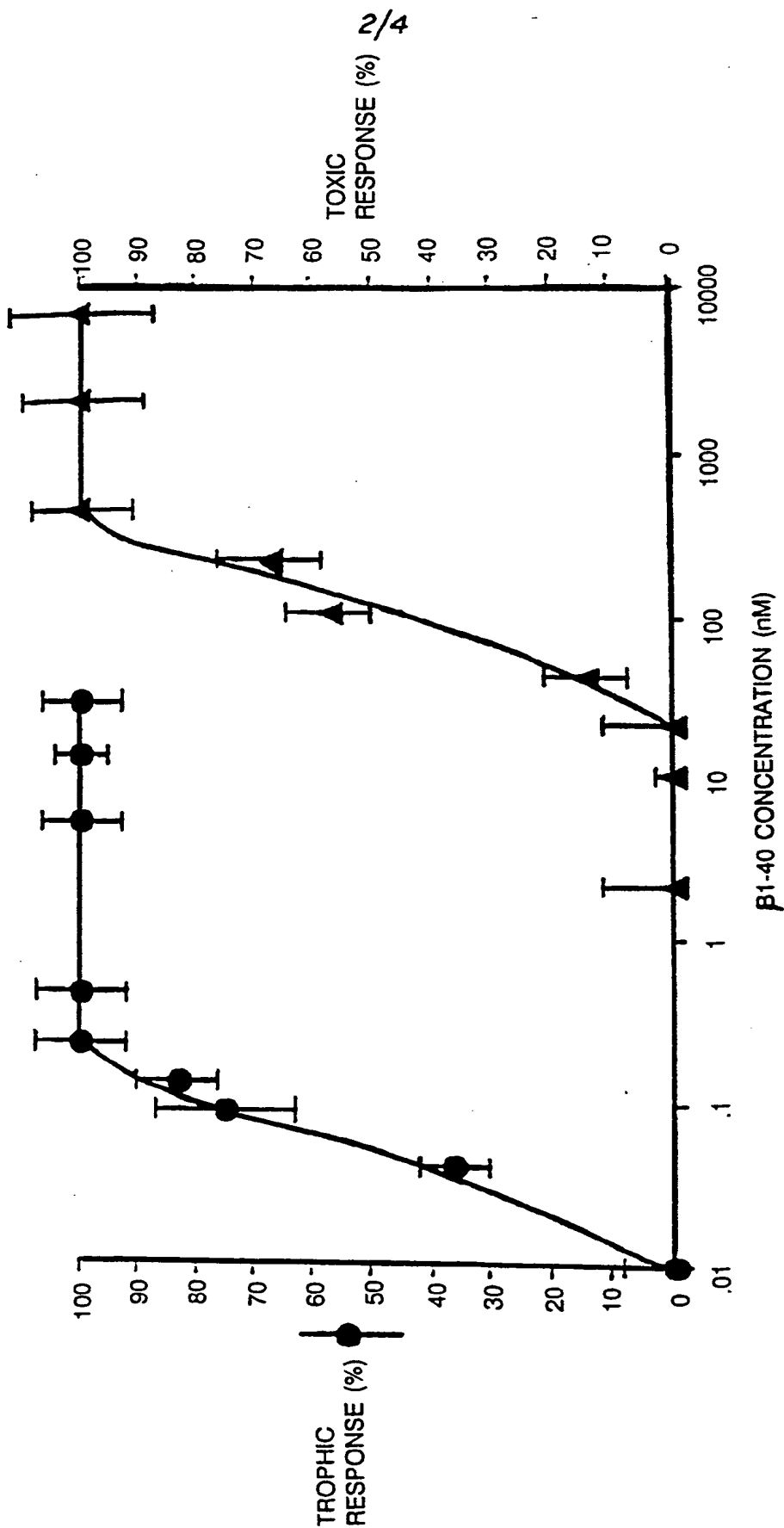
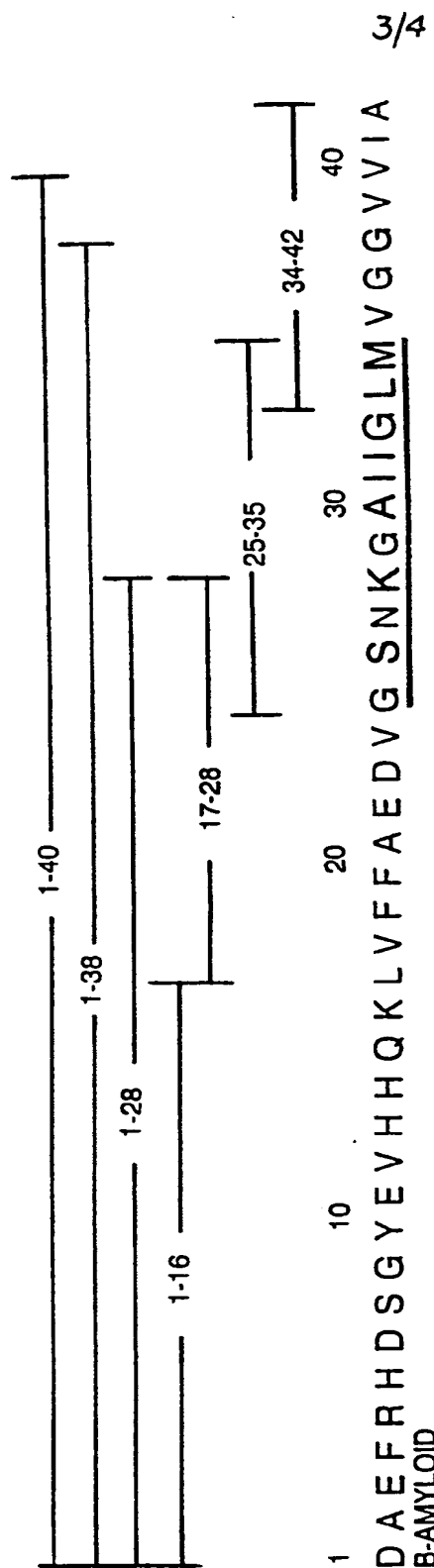


FIG. 2

SUBSTITUTE SHEET



E P S K D A F I G L M	Eledoisin
D M H D F F V G L M	Neurokinin B
H K T D S F V G L M	Neurokinin A
D V P K S D Q F V G L M	Kassinin
R P K P Q Q F F G L M	Substance P

FIG. 3

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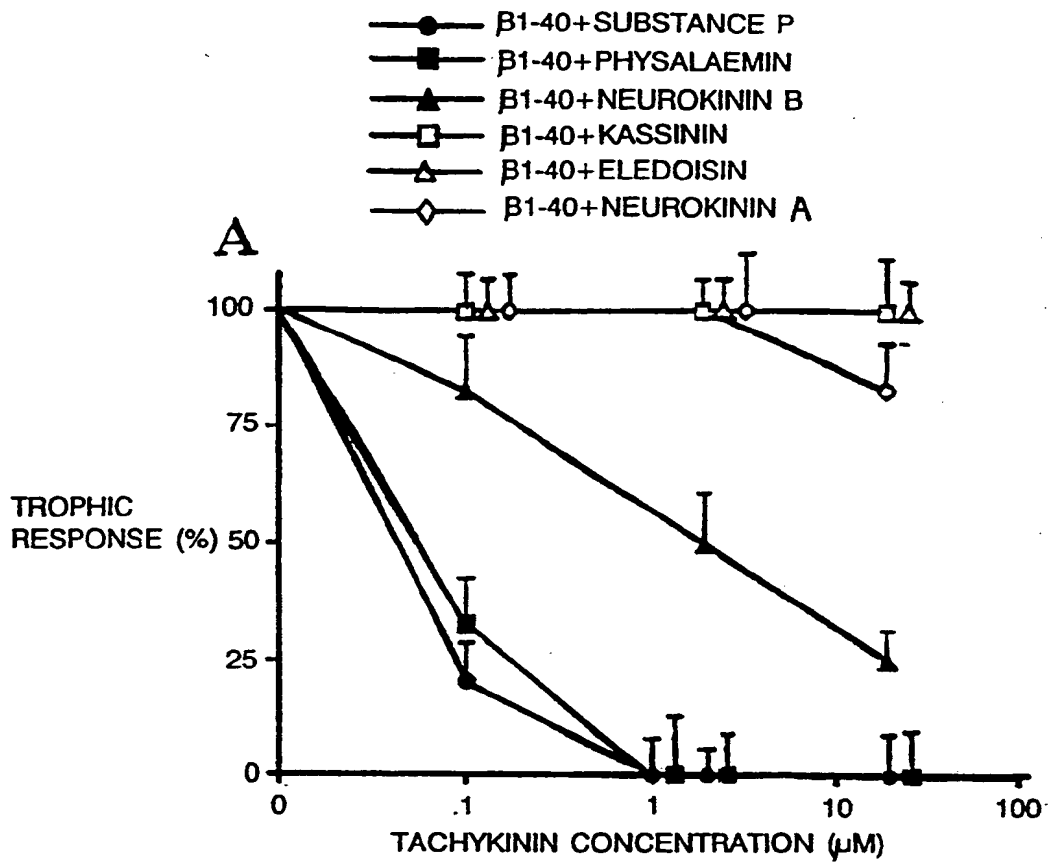


FIG. 4a

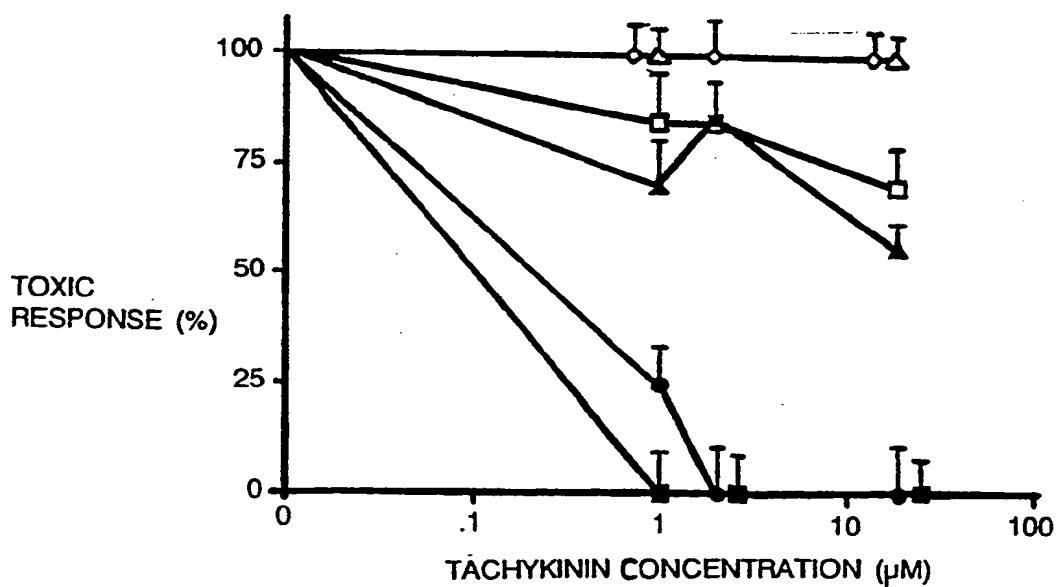


FIG. 4b

SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05323

## I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/42; C07K 7/22, 7/06; G01N 33/50

U.S.Cl: 514/15; 530/327; 436/501

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System	Classification Symbols
U.S.Cl;	514/14, 15, 18; 530/327, 328, 331, 839; 436/501, 811

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

APS, search terms: substance P, tachykinin, Alzheimer ? Down ? (W) syndrome ?  
Biosis, Medline, search terms: substance P. Tachykinin, Alzheimer ? Down ?  
(W) syndrome ?

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X, E	Proceedings of the National Academy of Sciences, vol. 88, issued August 1991, Kowall et al, "An <u>in vivo</u> model for the neurodegenerative effects of $\beta$ amyloid and protection by substance P", pages 7247-7251, see page 7249, first column, second paragraph.	1-5
X, P	Science, vol. 250, issued 12 October 1990, Yankner et al, "Neurotrophic and effects of amyloid $\beta$ protein: reversal by tachykinin neuropeptides", pages 279-282, see table 1, p 281.	1-5
Y	US, A, 4,728,605 (Fudenberg et al) 01 March 1988, see column 12, lines 37-50.	1-5
A	US, A, 4,059,693 (Stewart) 22 November 1977.	

\* Special categories of cited documents: \*\*

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

07 October 1991

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

07 NOV 1991

Signature of Authorized Officer

Stephen Walsh



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 3,862,114 (Scandrett) 21 January 1975.	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See Attached

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-5 (specie a)

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Serial No. PCT/US91/05323  
Art Unit 189B

Attachment to Form PCT/ISA/210/Part VI

Observations where unity of invention is lacking

### Itemized summary of claims groupings

The claims present mutually exclusive independent inventions as follows:

I. Claims 1-12, drawn to a method of treatment or prophylaxis, classified in Class 514, subclasses 15-18.

Note that the following are independent and distinct species pertinent to the invention of Group I where a) is the first species and will be searched with claims 1-5 in the event that no other fees are paid. Note that a search of any other additional species within Group I requires payment of additional fees.

- a)the agonist is Substance P;  
b)the agonist is physalaemin;  
c)the agonist is a fragment of Substance P (claims 6-9);  
d)the agonist is a Substance P derivative (claim 10);  
e)the agonist is a cyclic Substance P derivative (claim 11);  
f)the agonist is neurokinin B or derivative (claim 12).

II. Claims 13-17, drawn to a method for identifying a potentially therapeutic compound, classified in Class 435, subclass 7.21.

Note that the following are independent and distinct species pertinent to the invention of Group II where g) is the first species and will be searched upon payment of the requisite fee for Group II. Note that a search of any other additional species within Group II requires payment of additional fees.

- g) the determining step comprises administering to a cell;  
h) the determining step comprises administering to a transgenic mammal (claim 17).

III. Claims 18-19, drawn to a method for identifying an antagonist of neurotoxicity, classified in Class 435, subclass 7.21.

(continued)

Serial No. PCT/US91/ 5323  
Art Unit 1898

IV. Claims 20-23, drawn to a method for identifying a compound useful for treatment of a disease, classified in Class 435, subclass 7.21.

5 Note that the following are independent and distinct species pertinent to the invention of Group IV where i) is the first species and will be searched upon payment of the requisite fee for Group IV. Note that a search of any other additional species within Group IV requires payment of additional fees.

10 i) accumulation is determined by histochemical staining or indirect staining (claims 21 and 23);

j) accumulation is determined by binding of a radiolabeled peptide (claim 22).

15 Reasons for holding lack of unity of invention

20 The inventions are distinct, each from the other, because the claimed methods are practiced with materially different substances in materially different process steps. PCT Rules 13.1 and 13.2 do not provide for multiple distinct methods within a single general inventive concept.